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Inhibition and labelling of the mitochondrial 2-oxoglutarate carrier by eosin-5-maleimide

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Unlike hydrophobic maleimides, eosin-5-maleimide and to a lesser extent other relatively polar maleimides inhibit the 2-oxoglutarate carrier of bovine heart mitochondria. The impermeable eosin-5-maleimide labels the 2-oxoglutarate carrier in intact mitochondria but not in submitochondrial particles. 2-Oxoglutarate protects the carrier against inactivation by eosin-5-maleimide and decreases the fluorescence associated with the purified protein. Other anions which are not substrates of the carrier have no protective effect. It is concluded that sulfhydryl groups essential for the activity of the 2-oxoglutarate carrier are located at the cytosolic face of the inner mitochondrial membrane. They appear to be present at the substrate-binding site and located in a hydrophilic environment.

2-Oxoglutarate carrier; Eosin-5-maleimide labeling; Transport; Mitochondria; (Bovine heart)

1. INTRODUCTION

The 2-oxoglutarate carrier catalyzes the transport of 2-oxoglutarate through the inner mitochondrial membrane by a strict 1:1 counterexchange with malate or some other dicarboxylates (for a review see [1]). We have recently isolated and functionally reconstituted the 2-oxoglutarate transport protein from both heart and liver mitochondria [2–4].

Whereas the functional properties of the OGC have been extensively investigated both in mitochondria and in proteoliposomes [1–3], the information on the structure of this carrier is negligible. In order to understand the molecular mechanism of the 2-oxoglutarate transport in mitochondria, it is important to investigate the role and the location of the essential functional groups of the carrier protein. In this respect it is only known that the OGC is inhibited by mersalyl and *p*-chloromercuribenzoate but not by *N*-ethylmaleimide [2,5]. Further studies on the OGC's sulfydryl groups have been hindered by their relatively low reactivity with mercurials as compared to other carrier proteins [5], and also by the impossibility to label the protein with radioactive *N*-ethylmaleimide.

Here we report the effect of an impermeable, fluorescent maleimide analogue EMA on the mitochondrial OGC. This hydrophilic maleimide inhibits and labels the OGC in mitochondria but not in submitochondrial particles. Oxoglutarate protects the carrier against inactivation and labelling by EMA.

2. MATERIALS AND METHODS

N-(4-dimethylamino-3,5-dinitrophenyl)maleimide was purchased from Serva, *N*-cyclohexylmaleimide and *N*-phenylmaleimide from the Nutritional Biochemicals Corporation (Cleveland, OH), eosin-5-maleimide, 4-maleimidylsalicylic acid and 5-maleimidylsalicylic acid from Molecular Probes (Eugene, OR). Other reagents were obtained as previously reported [3,6].

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Abbreviations: EMA, eosin-5-maleimide; OGC, 2-oxoglutarate carrier; Pipes, 1,4-piperazinediethanesulphonic acid

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Mitochondria and inside-out submitochondrial particles from bovine heart were prepared as described in [7,8], respectively. Solubilization of mitochondria and submitochondrial particles, purification of the OGC by hydroxyapatite/celite chromatography and reconstitution of the hydroxyapatite/celite eluate in liposomes prepared in the presence of 1 mM 2-oxoglutarate were carried out as described previously [2,3]. The proteoliposomes were incubated with the indicated maleimides in 20 mM NaCl, 10 mM Pipes, 1 mM 2-oxoglutarate and 1 mM EDTA, pH 7.0. After 3 min at 25°C, the reaction was stopped by the addition of 10 mM dithioerythritol. After a further 2 min, transport was initiated by adding carrier-free [^{14}C]oxoglutarate and terminated 8 min later by the addition of 10 mM phthalonate, a powerful inhibitor of the OGC. In the control samples phthalonate was added together with the labelled substrate at time zero. The activity was calculated by subtracting the control values from the experimental samples [9].

Modification of the OGC by maleimides in freshly prepared mitochondria or submitochondrial particles was performed at 0°C in a medium containing 250 mM sucrose, 1 mM EGTA and 10 mM Tris-HCl, pH 7.4, at a protein concentration of 10 mg/ml with 250 μM EMA for 45 min in the dark. The reaction was terminated by the addition of 10 mM dithioerythritol. After an additional 2 min the labelled membranes were washed twice in the same medium. The OGC was then purified from both mitochondria and submitochondrial particles and the activity of 2-oxoglutarate transport was measured in reconstituted liposomes.

Polyacrylamide slab gel electrophoresis was performed as in [2]. After electrophoresis, fluorographs were obtained by illuminating the slab gel with UV light and photographing the fluorescent emission through cutoff filters. Subsequently, the gel was stained by the silver nitrate method [10]. Protein was determined by the Lowry method modified for the presence of Triton [11]. Glutathione was determined as in [12].

3. RESULTS

The effects of *N*-ethylmaleimide and EMA on the reconstituted oxoglutarate/oxoglutarate exchange activity are shown in fig.1. Whereas *N*-ethylmaleimide did not significantly inhibit the OGC protein, EMA was a powerful inhibitor of oxoglutarate transport in proteoliposomes. Thus, half-maximal inhibition was achieved with 26 μM EMA. In other experiments (not shown) the effectiveness of other maleimides in inhibiting the reconstituted oxoglutarate transport activity was tested. Like *N*-ethylmaleimide, the hydrophobic *N*-phenylmaleimide and *N*-cyclohexylmaleimide had very little, if any, inhibitory effect. The relatively polar 4-maleimidylsalicylic acid, 5-maleimidylsalicylic acid and *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide inhibited oxoglutarate transport to some extent (27–45% at a concentration of 1 mM). Among the maleimides tested, the more polar EMA was the most effective inhibitor of oxoglutarate transport (92% at 1 mM).

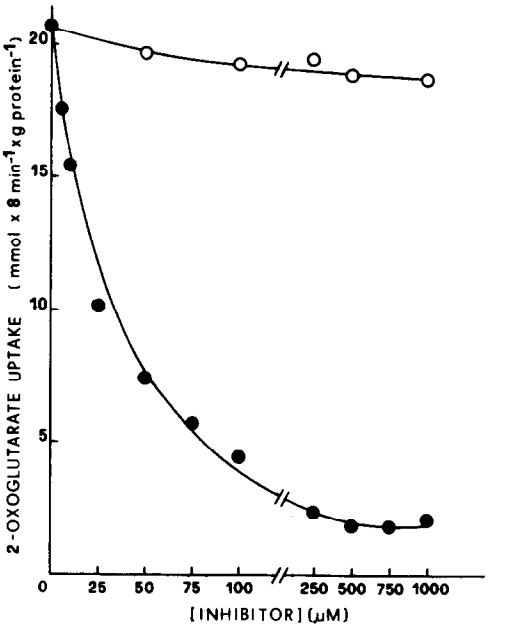

Fig.1. Inhibition by EMA of the reconstituted oxoglutarate transport activity. The reconstituted liposomes were incubated with EMA (●) or *N*-ethylmaleimide (○) at the concentrations indicated.

Fig.2 shows the SDS-polyacrylamide gel electrophoresis and the fluorography of Triton X-114 extracts from bovine heart mitochondria and submitochondrial particles which had been incubated with 250 μM EMA at 0°C for 45 min. Under these conditions, EMA did not permeate the inner mitochondrial membrane since it did not decrease the content of matrix glutathione. In both mitochondrial and submitochondrial extracts several proteins were labelled by EMA (fig.2B, lanes 2 and 4). Among these, a 30 kDa protein was highly labelled in both preparations. This presumably corresponds to the ADP/ATP carrier, which has been found to bind EMA [13–15]. From the results shown in lanes 2 and 4 it was not possible to conclude whether or not EMA was bound to the OGC, because this carrier is present in minute amounts compared to other proteins and has an M_r slightly higher than that of the ADP/ATP carrier [2]. To ascertain whether the OGC could be la-

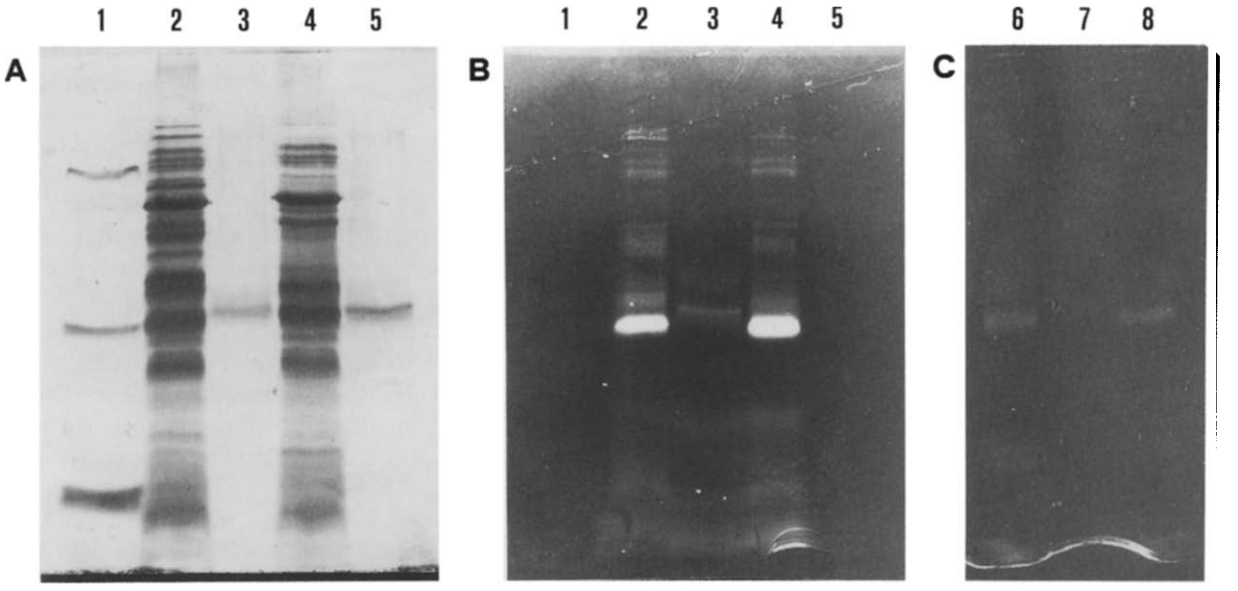

Fig.2. Differing ability of EMA in labelling SH groups of the OGC in mitochondria and in submitochondrial particles. SDS-polyacrylamide gel electrophoresis (A) and fluorography (B) of Triton X-114 extracts obtained from mitochondria (lanes 2) or from submitochondrial particles (lanes 4), and of the purified OGC obtained from EMA-labelled mitochondria (lanes 3) or from EMA-labelled submitochondrial particles (lanes 5). C shows the fluorography of the purified OGC obtained from intact mitochondria incubated with EMA in the presence (lane 7) and absence (lane 6) of 5 mM oxoglutarate. In lane 8, 5 mM 2-oxoadipate was present instead of oxoglutarate. Marker proteins (bovine serum albumin, carbonic anhydrase, cytochrome *c*) are shown in lanes 1.

belled by EMA, the OGC protein was isolated from both intact mitochondria and submitochondrial particles pretreated with 250 μM EMA. As shown in fig.2B, lane 3, the OGC isolated from mitochondria was labelled by EMA. In contrast, no labelling of the OGC isolated from submitochondrial particles was observed (fig.2B, lane 5). Accordingly the activity of the reconstituted OGC isolated from EMA-labelled mitochondria was inhibited by 75%, whereas that of the carrier derived from EMA-labelled submitochondrial particles was not affected (not shown). Increasing the concentration of EMA to 1 mM and the time of incubation to 60 min also failed to induce labelling of OGC in submitochondrial particles; the labelling of the carrier protein in mitochondria, on the other hand, was time- and concentration-dependent (not shown).

In order to obtain information on the site of the OGC to which EMA binds, the effect of oxoglutarate on inactivation and labelling of the OGC by EMA in intact mitochondria was investigated. Fig.3 shows that the inhibition of oxoglutarate transport activity by 250 μM EMA was

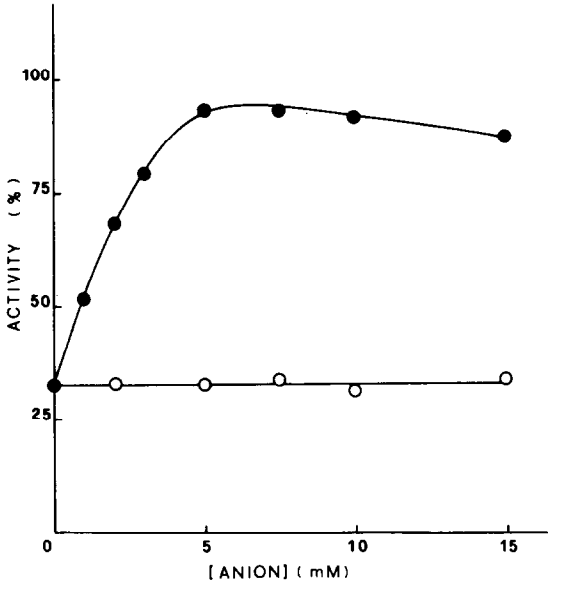

Fig.3. Effect of oxoglutarate and 2-oxoadipate on the inhibition of the OGC by EMA in intact mitochondria. Oxoglutarate (●) or 2-oxoadipate (○) was present at the concentrations indicated during the incubation of the mitochondria with 250 μM EMA. The activity was measured in liposomes reconstituted with purified OGC.

progressively decreased by the presence of increasing concentrations of oxoglutarate during the incubation of the mitochondria with EMA. 5 mM oxoglutarate nearly completely protected the OGC against inhibition by EMA. The specificity of the protection of the OGC against EMA inactivation was investigated by testing the effect of several other anions. As shown in fig.3, 2-oxoadipate up to 15 mM had no protective effect. Similarly glutarate, 2-oxomalonate, ADP, citrate, sulphate and phosphate had virtually no effect on the inhibition of the OGC by EMA (not shown). In contrast, malate and malonate, which are known substrates of the OGC [6], caused a significant protection. The observed protection by oxoglutarate against inhibition by EMA was accompanied by a decreased labelling of the carrier. Thus no fluorescence associated with the OGC purified from EMA-labelled mitochondria was observed when 5 mM oxoglutarate was present during the incubation of the mitochondria with EMA (fig.2C, cf. lane 7 with 6). In the presence of 2-oxoadipate, on the other hand, the intensity of the label bound to the OGC was not reduced (fig.2C, lane 8).

4. DISCUSSION

The results presented in this paper show that, unlike *N*-ethylmaleimide, the highly polar EMA strongly inhibits the OGC of bovine heart mitochondria. The extent of inhibition caused by the maleimides tested seems to correlate with their hydrophilicity since the relatively polar *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide and salicilylmaleimides also cause some inactivation of the OGC, whereas the hydrophobic maleimides have no effect. These results are in contrast to the effect of maleimides on the phosphate and the ADP/ATP carrier which are inhibited by both *N*-ethylmaleimide and EMA [13–15]. The differential behaviour of the OGC towards polar and apolar maleimides, respectively, suggests that the environment of the essential sulfhydryl groups of the OGC has a hydrophilic character.

The fact that the impermeable SH-binding reagent EMA inhibits and labels the OGC in intact mitochondria, but not in submitochondrial particles, clearly indicates that the essential EMA-reacting SH groups of the OGC are located at the cytosolic face of the inner mitochondrial membrane. This asymmetry of the OGC seems to be preserved even after reconstitution of the protein into the liposomes, since EMA also almost completely inhibits the reconstituted carrier (fig.1). In intact mitochondria the OGC activity is completely inhibited by 0.5 mM EMA (not shown).

Another structural feature of the OGC can be derived from the experiments presented in this paper. Oxoglutarate protects the OGC from inhibition and labelling by EMA whereas other anions, which are not substrates of the OGC, show no protective effect. These results can be interpreted to indicate that the SH groups reacting with EMA are located in the vicinity of the substrate-binding site.

In conclusion, EMA appears to be a suitable tool for the characterization of essential sulfhydryl groups present at, or near, the substrate-binding site of the OGC, which faces the cytosol. Experiments are now in progress to locate the EMA-reacting site(s) within the amino acid sequence of the OGC.

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